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(71) Applicants and
(72) Inventors: **HERRMANN, Björn** [SE/SE]; Skrindvägen 21, S-756 47 Uppsala (SE). **KIRSEBOM, Leif** [SE/SE]; Vreta Strandväg 20, S-755 91 Uppsala (SE). **STOLT, Pelle** [SE/SE]; c/o Leif Kirsebom, Vreta Strandväg 20, S-755 91 Uppsala (SE).

(74) Agent: **DR LUDWIG BRANN PATENTBYRÅ AB**; Box 1344, Drottningg. 7, S-751 43 Uppsala (SE).

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(54) Title: A METHOD FOR DETECTION OF PATHOGENIC ORGANISMS

(57) Abstract: The present invention relates to a method for detection of pathogenic organisms wherein the method includes differentiation between species. The method is especially suitable to detect and to diagnose infection by pathogenic organisms which are hard and/or laborious to detect with conventional methods. The method relies upon analysis of specific variable regions of the RNase P RNA gene, namely the P3 and/or P19 region(s).

Title: A method for detection of pathogenic organisms

Field of the invention

The present invention relates to a method for detection of pathogenic organisms wherein the method includes differentiation between species. The method is especially suitable to detect and to diagnose infection by pathogenic organisms which are hard and/or laborious to detect with conventional methods. The method relies upon analysis of specific variable regions of the RNase P RNA gene.

Background of the invention

RNase P is an enzyme present in all living cells. It catalyses the removal of 5' leader sequences from tRNA precursor molecules. In bacteria, RNase P consists of an RNA molecule of some 400 nt in length (11, 28) and a small (about 120 aa) protein (33). In the division bacteria, the RNA moiety has been shown to function as an efficient catalyst *in vitro* (12); hence at least in these organisms, RNase P is a ribozyme (an RNA molecule catalysing chemical reactions). Bacterial RNase P RNAs have been separated into two main structural classes. Type A is the most common structural class and type B is found in the low G+C Gram-positive Bacteria (50). The secondary structure of RNase P RNA has been characterised for many bacterial lineages and variation among the helices provides useful phylogenetic information (51).

The RNase P RNA gene sequences are not very well preserved between bacterial groups, (5) but within a genus, genes can be quite similar. Several hundreds of RNase P RNA sequences are present in the RNase P RNA database ((http://jwbrown.mbio.ncsu.edu/RNaseP/home.html).

The order *Chlamydiales* is a group of obligately intracellular bacteria which have a unique developmental cycle and pathogenicity. They are parasites of humans and a wide variety of animals. Species in the *Chlamydiaceae* family have recently been reclassified into two genera, *Chlamydia* and *Chlamydophila*, that include nine species (43). In addition, new families now also belong to *Chlamydiales*, and they include *Parachlamydiaceae* and *Simkaniaceae* (43). The type species of *Parachlamydiaceae* is *Parachlamydia acanthamoebae*, a symbiont of the amoebae *Acanthamoeba castellani* and an occasional pathogen of people who acquire this amoebae (34). *Simkania negevensis* is the type species of *Simkaniaceae* and, like many other chlamydiae, also causes human infection (56, 57, 61).

It has previously been shown that the RNase P RNA genes in the genus *Clamydia* differ sufficiently between species to be useful as a diagnostic

tool (13); thus the gene is potentially useful for strain differentiation. The differences between sequences also give hints to which parts of the molecule are important for catalytic activity, complementing mutational and structural studies.

Another important family of pathogens, where fast and sensitive diagnostic methods are vital, are the mycobacteria. Traditional diagnostic methods have relied on the demonstration of acid-fast bacilli in clinical samples following cultivation. This is reliable but time consuming, since slow-growing species such as *Mycobacterium tuberculosis* may need six to eight weeks to form a sufficiently large population. In the last few years, many PCR-based detection assays have been developed based on eg the *hsp60* gene (16) or the variable interspersing region between the 16S and 23S rRNA genes (15, 24, 30) and this trend continues.

The RNase P RNA gene sequence from *M. tuberculosis* is known (6) as well as that from *M. bovis* BCG and *M. leprae* . The *M. bovis* sequence is identical to that from *M. tuberculosis*, while there are differences to *M. leprae*. The regions in the RNase P RNA gene which have been indicated by other means as important for catalytic activity, were almost totally conserved between the mycobacteria. The close relationship within microbial genuses, such as mycobacteria, has rendered differentiation between species of the same genus very difficult or impossible.

Summary of the invention

The present invention solves the problem of differentiation between species within the same genus, such as mycobacteria and chlamydia. Furthermore, the present invention solves the problem of detecting pathogens which are hard and/or laborious to detect with conventional methods.

The inventive method may in principle be used for diagnosing infection by any kind of pathogenic organism.
Thus the pathogens include archaebacteria and eubacteria. The latter contains as its principal groups gliding bacteria, spirochetes, rigid bacteria and mycoplasmas. Rigid bacteria include actinomycetes and simple unicellular bacteria. The latter group consists of obligate intracellular parasites and free-living bacteria. Among the free-living variants there are (1) gram-positive bacteria in which group there are (a) cocci, (b) non-sporulating rods, (c) sporulating rods that can be further subdivided into obligate aerobes and obligate anaerobes; and (2) gram-negative bacteria in which there are (a) cocci, (b) non-enteric rods with spiral forms and straight rods, and (c) enteric rods with facultative anaerobes, obligate aerobes and obligate anaerobes. For specific bacteria species see further Medical Microbiology, (Brooks et al, eds., 19th ed. (1991), Prentice-Hall International, USA).

The pathogens include also fungi, including pathogenic yeasts and molds. Examples are Apergillus, Candida, Absidia, Mucor, Rhizopus, Cryptococcus, Hisoplasma, Blastomyces, Coccidiodes, Paracoccidiodes, Sporotrichosis, Chromoblastomycosis, Mycetoma, Microsporum, Trichophyton and Epidermophyton.
Other pathogens that may be diagnosed may be found among the protozoa and algae.

Of course, the method of the invention can also be used for detection of non-pathogenic bacteria.
The following bacteria are especially interesting for the purposes of the present invention: Bacteria of phylum II – Green bacteria; Phylum III – Deinobacteria (Thermus/Deinococcus); Phylum IV - Spirochetes; Phylum VI – Gram negative anaerobes and gliding bacteria (Bacteroides/Flavobacterium); Phylum VIII - Chlamydia; Phylum IX – Gram positives with the three lineages: lineage A ("Gram negatives"), lineage B (G+C-rich bacteria), lineage C (G+C-poor bacteria); Phylum X - cyanobacteria; Phylum XI - proteobacteria with the five lineages alpha, beta, gamma, delta and "E".

The present inventors detected differences in the RNase P RNA gene which were sufficient to enable species determination.

In a first aspect, the invention relates to a method for detection of pathogenic organisms including inter-species differentiation, comprising using the P3 and/or P19 hypervariable region(s) of the RNase P RNA gene as a diagnostic target.

The purpose of the method of the invention may be, for example, diagnose of infection caused by pathogenic bacteria and epidemic investigation of the spreading of drug resistant bacteria.

Preferably, the region(s) is/are amplified, such as by PCR (Polymerase Chain reaction) and sequenced or otherwise fingerprinted for species identification, such as by heteroduplex analysis, size determination, RFLP (Restriction Fragment Length Polymorphism), melting point determination etc.

In a second aspect the invention relates to a method for detection of bacteria including inter-species differentiation, comprising amplification of nucleic acids of hypervariable region(s) of the RNase P RNA gene from the pathogens; forming a heteroduplex with related nucleic acid; and analysis thereof.

An example of a method according to the invention involving a heteroduplex analysis, comprises of an amplification reaction, a

hybridisation step and a non-denaturing gel electrophoresis analysis. The whole process can be carried out in less than 24 hours.

In a third aspect, the invention relates to use of the P3 and/or P19 variable regions(s) in the RNAse P RNA gene as a drug target in the production of a medicament for the treatment of microbial infection.

Detailed description of the invention

The invention will now be described in greater detail in relation to two non-limiting examples.

EXAMPLE 1: MYCOBACTERIA

MATERIALS AND METHODS

Bacterial strains. Mycobacteria used in this study are listed in Table 1 below. Clinical strains were already typed by 16S RNA gene sequencing in their respective institutes of provenance.
The RNase P RNA sequences for *M. tuberculosis*, *M. bovis* BCG and *M. leprae* were retrieved from the GenBank sequence database.

Table 1

Species	Strain description	Provenance
M. gastri	ATCC 15754	ATCC
M. kansasii	ATCC 12748	ATCC
M. intracellulare	D673	Trudeau Mycobacterial Collection
M. xenopi	ATCC 19276	ATCC
M. smegmatis	mc^2 155	Ref. 26
M. avium	D702	Trudeau Mycobacterial Collection
M. marinum	clinical strain	Research Centre Borstel

M. fortuitum	clinical strain	Research Centre Borstel
M. malmoense	clinical strain	Research Centre Borstel
M. paratuberculosis	6783	G-F Gerlach School of Veterinary Medicine, Hanover Germany
M. gordoniae	clinical strain	Research Centre Borstel
M. celatum	clinical strain	Research Centre Borstel

PCR amplification of the RNase P RNA genes. Based on the published sequences of *M. tuberculosis* and *M. leprae*, (GenBank Accession Numbers Z70692 and L78818 respectively) a primer pair was designed which hybridised close to the ends of the gene. The forward primer tbf (5' CGGATGAGTTGGCTGGGCGG 3') and reverse primer tbr (5' GTTGGCCTGTAAGCCGGATT 3') both show one mismatch to the *M. leprae* sequence. Using this primer pair, the RNase P gene could be amplified from all mycobacteria tested. Most reactions were run on untreated mycobacteria from cultures, without previous isolation and purification of chromosomal DNA. PCR was done in 50 µl reactions in a Rapidcycler capillary PCR apparatus (Idaho technology, Idaho Falls USA) with the following parameters: 94°C 10"; 50°C 10" 72°C 15".

Sequencing. PCR products were purified over 1 per cent agarose gels to remove the primers. Approximately 1/20 of the purified DNA was used for automated sequencing with the same primers as were used in the amplification reaction. Sequencing was done on an Applied Biosystems model 310 capillary sequencer.

Heteroduplex analysis. For heteroduplex analysis of the RNase P P3 loop region, DNA was amplified (50 µl reactions) with the oligonucleotides tbf and 280r (5' CTTGCTTGCCCTCCCTTTGCC 3') which give a product of about 250 bp. The products were gel purified and approximately 1/10 of the products used in each analysis. DNA was mixed with equal amounts of DNA from *M. tuberculosis*, heated to 95 °C for 1' and allowed to cool to room temperature. The products were separated on 10 per cent non-

denaturing polyacrylamide gels run at 15 mA for 14 hrs. Bands were visualised by silver staining.

Silver staining of DNA gels. Gels were fixated in 10 per cent ethanol for 5', and incubated in 1 per cent nitric acid for 5'. Staining was in a 1 mg/ml solution of silver nitrate for 30'. Bands were developed in a sodium carbonate/formaldehyde solution (15 g anhydrous sodium carbonate and 300 µl 37 percent formaldehyde in 500 ml water) until clearly visible. The reaction was stopped with 10 percent acetic acid.

RESULTS

The results from Example 1 will be presented below in association with the accompanying drawings, Figs 1-5:

FIG. 1. Sequence alignment of the RNase P RNA genes from mycobacteria. Dashes indicate sequence identities; stars mark bases missing in a sequence.

FIG. 2. Secondary structure of *M. tuberculosis* RNase P RNA. Regions diverging between mycobacterial species are shaded. Minor variable regions are not shown.

FIG. 3. Sequence alignment of the RNase P RNA gene from *M. gastri* (identical sequence from two different strains) and six strains of *M. kansasii.* Bases unique to the *M. gastri* sequence are boldfaced.

FIG. 4 A. Heteroduplex analysis of the first 280 bp from RNase P RNA gene regions from different mycobacteria. DNA from diverse mycobacterial species was hybridised with *M. tuberculosis* DNA and the resulting duplexes separated on a 10 per cent polyacrylamide gel. Lane 1, control DNA from *M. tuberculosis*; lane 2, *M. avium*; lane 3, *M. intracellulare*; lane 4, *M. malmoense*; lane 5, *M. celatum*; lane 6, *M. kansasii*; lane 7, *M. vaccae*; lane 8, *M. xenopii.*

FIG. 4B. Heteroduplex analysis of DNA amplified from clinical samples of bacteria believed to be either *M. intracellulare* or *M. avium.* Lane 1, control DNA from *M. tuberculosis*; lanes 2, 3, 6, DNA from suspected *M. avium*; lanes 4, 5, 7, DNA from suspected *M. intracellulare*; lane 8, control DNA from *M. avium*; lane 9, control DNA from *M. intracellulare.*

FIG. 5. Suggested structure for the P18 loop of RNase P from mycobacteria, based on sequence variations within the genus. The sequences are from A) *M. tuberculosis*; B) *M. smegmatis*; C) *M. marinum*

In PCR amplifications of the RNase P RNA gene from mycobacteria using the oligonucleotides tbf and tbr, each reaction yielded a single fragment in the range of 387 (*M. celatum*) to 428 (*M. tuberculosis*) bp depending on mycobacterial species.

Using these oligonucleotides it was possible to amplify the RNase P RNA genes from all mycobacteria tested, using untreated cells from culture or plates. No previous purification of chromosomal DNA was necessary.

An alignment of the sequences (Fig.1) shows very well conserved nucleotide sequences along most of the gene, with the exception of three major regions where the similarities break down and no adequate alignment is possible. The overall similarity of the genes was between 80 and 85 per cent. This is not a very informative figure however, since the similarity within the well conserved regions is close to 100 per cent, while the divergent regions are too dissimilar to be aligned at all.

If the divergent regions are localised on a two-dimensional representation of the RNase P RNA molecule (Fig.2), it is conspicuous how most of the differences fall into regions which belong to ends of stem-loop structures. The major divergent regions are the P3, P16 and P19 loops. In addition, there is a deletion in the P12 loop in *M. smegmatis* and *M. fortuitum*, comprising several unpaired nucleotides of this loop. The major interspecies differences are within the P3 and P19 loops.

All species analysed had specific RNase P RNA gene sequences. Several closely related species could be differentiated on basis of the RNase P RNA sequences. The members of the MAI complex *M. avium* and *M. intracellulare* differed in several positions, including a deletion in the P19 loop in *M. avium* (Fig. 2). The very closely related (subspecies) *M. paratuberculosis* and *M. avium* were indistinguishable on basis of RNase P RNA gene sequences.

As far as our analysis went, within a species, sequences are identical. We sequenced clinical samples from all members of the *M. tuberculosis* complex (*M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum, M. microti* and *M. tuberculosis* ssp asiaticum) without detecting any deviations from the published *M. tuberculosis* sequence (6). Thus, members of this group could not be differentiated on the basis of RNase P RNA gene differences.

The possibility of sequence differences between different serovars of mycobacteria was investigated for some species. In the case of *M. avium* and *M. intracellulare*, the RNase P RNA gene from five clinical isolates (animal) of *M. avium* and five *M. intracellulare* (human) was amplified and sequenced. No differences between serovars could be detected.

The one species where heterogenous RNase P RNA gene sequences were observed between different serovars was *M. kansasii* (Fig. 3). The differences were mainly C-to-T transitions. Two strains of *M. gastri* were sequenced as well, but in this species the gene sequences were identical between isolates. In four positions, all RNase P RNA gene sequences from *M. kansasii* strains differed from those of *M. gastri*. Three of these differences were C-to-T transitions, while the fourth was a C-to-A transversion.

The interspecies differences found in the P3 and P19 loops were considered to be large enough for an attempt at a simple diagnostic application through heteroduplex analysis. The hypervariable P3 region was chosen for this analysis and amplified using the tbf and 280r oligonucleotides. Each PCR reaction yielded single bands of about 250 bp. The region from *M. tuberculosis* was used as a standard and was mixed in equal amounts with the products from different species. After separating the fragments on non-denaturing polyacrylamide gels, the bands were silver stained. There were clear differences in the heteroduplex pattern between all species tested (Fig. 4A).

This analysis was taken further by applying it to clinical samples (from the Swedish Institute of Infectious Disease Control) which had or had not been previously typed. On the resulting gel (Fig. 4B) the samples in lanes 2, 3 and 6 can be attributed to *M. avium* (compare to lane 8), while samples 4, 5 and 7 seem to be *M. intracellulare* (compare to lane 9). Sequencing the RNase P RNA gene in each case confirmed the results obtained from heteroduplex analysis.

DISCUSSION

Compared with the case of *Clamydia*, the RNase P genes from mycobacteria are better preserved between species, with overall similarities of 80-85 per cent. This overall value is misleading, however, since most differences cluster in specific regions, where the variability is of a degree that makes an unambiguous alignment impossible. All mycobacterial RNase P RNAs show a P15-P17 region of the *Clamydia* and cyanobacteria type, (13, 31) which is hardly surprising given the close relatedness of the organisms.

All species studied had their own conspicuous sequence characteristics. Within a species, no differences were seen except for *M. kansasii*, a species which has on other grounds been described as heterogeneous (1, 14, 32). Even though several of the variant positions were shared between *M. kansasii* and *M. gastri*, there are enough *M. kansasii*-specific bases in the gene to enable differentiation from the closely related *M. gastri* through microsequencing or heteroduplex analysis. *M. kansasii* is

an important cause of pulmonary disease resulting from non-tuberculous mycobacteria.

The combination of very well conserved regions and hypervariable sites in the RNase P RNA gene sequence enables fast typing of unknown mycobacterial samples. Oligonucleotides will hybridise with perfect or close to perfect match to conserved regions allowing for a reliable amplification of the variable parts in between.

The *M. avium* complex (MAI) which includes *M. avium* and *M. intracellulare*, is a major opportunistic infection in AIDS patients (21, 22). Differentiating between the members of this complex requires molecular methods and our heteroduplex analysis of PCR products from the RNase P RNA gene offers a rapid and fairly inexpensive alternative to current methods (7, 8, 10, 18, 19, 23, 27, 29).

Sequence variances in the RNase P RNA gene between species can also yield clues to the molecular structure of the RNA. In the case of the mycobacteria (Fig. 2), almost all variances in the sequences were in unpaired regions, or in structures which seem unimportant for catalytic activity *in vitro.*

For instance, in two species, *M. smegmatis* and *M. fortuitum*, the bases 160-164 corresponding to the end of the P12 loop, (Fig. 2) are not present in the RNase P RNA gene sequence. The P12 loop is missing in the gene from some other organisms, such as *Mycoplasma fermentans* (4, 25) and thus does not seem necessary for ribozyme activity.

The alignment also supports conclusions about RNase P structure drawn from other experiments. Suggested important regions such as the motif bp 75-85 and pairing nt 409-417 (Fig. 2) are preserved throughout all analysed mycobacterial species. In all probability, these two regions base pair, since matching sequences are well conserved between organisms. The most variable regions between mycobacterial species were the P3 and P19 loops respectively. The P19 structure is not necessary for RNase P activity *in vitro* (25) and there is considerable variability in the P3 loop between organisms, but their role *in vivo* is unclear.

The suggested structure of *M. tuberculosis* RNase P RNA could still be improved upon with the help of sequence variations between species. A structure which is believed to be of importance is the P18 stem-loop, (the region nt 330-351) which has a well-preserved stem in the *Escherichia coli* and *Clamydia* RNase P RNAs. The suggested mycobacterial structure has a far less convincing stem structure. (See Fig. 2 , which is based on the old structure prediction.) However, in *M. smegmatis* and *M. marinum* there are variations from the consensus sequence. (Fig.1) A slightly different stem-loop structure would accommodate for these exchanges

while keeping a consensus secondary structure intact, at the same time allowing for a more convincing base-paring pattern (Fig. 5). This also strengthens the argument for the importance of this stem-loop for RNase P function.

EXAMPLE 2: CHLAMYDIA

MATERIALS AND METHODS

Bacterial strains. DNA of analysed organisms were released by standard Proteinase K treatment of cell culture grown organisms and phenol extracted or were provided as purified DNA-preparations (Table 2).

Table 2. Strains, host of origin, references, sources and accession numbers

Superscript 'T' indicates the type species

Strain	Host of origin	Reference	Source*	Accession no
Chlamydophila psittaci				
6BC^T (VR-125^T)†	Psittacine	Golub & Wagner (1947)	Storey	AJ012169
GD	Duck	Illner (1960)	NADC	
NJ1	Turkey	Page (1959)	NADC	
WC	Cattle	Page (1967)	NADC	
VS225	Psittacine		NADC	
360	Duck		Storey	
N352	Duck	Richmond *et al.* (1982)	Storey	
Cal10	Human	Francis & Magill (1938)	Storey	
CP3 (VR-574)	Pigeon	Page & Bankowski (1960)	NADC	
M56 (VR-630)	Muskrat	Spalatin *et al.* (1966)	NADC	
Chlamydophila abortus				
B577^T (VR-656^T)	Ovine, abortion	Perez-Martinez & Storz (1985)	Denamur	AJ131092
EBA	Bovine, abortion	Perez-Martinez & Storz (1985)	NADC	
OSP	Ovine	Andersen & Van Deusen (1988)	NADC	
EAE/Lx	Ovine, abortion		Storey	
AB7	Ovine, abortion	Rodolakis *et al.* (1989)	Rodolakis	
OC1	Ovine, conjunctivitis	Rodolakis *et al.* (1989)	Rodolakis	
AV1	Bovine, abortion	Rodolakis *et al.* (1989)	Rodolakis	
AC1	Caprine, abortion	Rodolakis *et al.* (1989)	Rodolakis	
iC1	Ovine	Rodolakis *et al.* (1989)	Rodolakis	
Chlamydophila felis				
FP Baker^T	Feline	Baker (1942)	NADC	AJ012171
Cello	Feline	Cello (1967)	Storey	
Pring	Feline	Wills *et al.* (1984)	Storey	
Chlamydophila caviae				
GPIC^T (VR-813^T)	Guinea pig	Murray (1964)	Storey	AJ012172
Chlamydophila pecorum				
E58^T (VR-628^T)	Bovine	McNutt & Waller (1940)	NADC	AJ012173
iB1	Ovine	Rodolakis *et al.* (1989)	Rodolakis	AJ131091
iC4	Ovine	Rodolakis *et al.* (1989)	Rodolakis	
AB10	Ovine, abortion	Rodolakis *et al.* (1989)	Rodolakis	
IPA (VR-629)	Ovine	Page & Cutlip (1968)	NADC	AJ131090
H3	Koala		Storey	AJ131089
Chlamydophila pneumoniae‡				
TW183^T (VR-2282^T)	Human	Grayston *et al.* (1989)	ATCC	AJ012174
CM1 (VR-1360)	Human	Black *et al.* (1992)	Black	
CWL011	Human	Black *et al.* (1992)	Black	
CWL029 (VR-1310)	Human	Black *et al.* (1992)	Black	
CWL050	Human	Black *et al.* (1992)	Black	
FML16	Human	Black *et al.* (1992)	Berdal	
P1	Human	Herrmann *et al.* (1996)	UHU	
JG915	Human	Herrmann *et al.* (1996)	UHU	
JG954	Human	Herrmann *et al.* (1996)	UHU	
TWAR 2023 (VR-1356)	Human	Chirgwin *et al.* (1989)	ATCC	
Chlamydia trachomatis				
A/Har-13^T (VR-571B^T)§	Human	Wang & Grayston (1962)	Persson	AJ131088
L1/440/LN (VR-901B)§	Human	Schachter & Meyer (1969)	Persson	AJ012175
Chlamydia suis				
S45^T (VR-1474^T)	Swine	Kaltenboeck *et al.* (1993)	NADC	AJ012176
R22	Swine	Rogers *et al.* (1993)	NADC	
Clamydia muridarum				
MoPn^T (VR-123^T)	Mouse	Nigg (1942)	NADC	AJ012177
SFPD	Hamster	Stills *et al.* (1991)	NADC	
Simkania negevensis				
Z^T (VR-1471^T)	Cell culture	Kahane *et al.* (1995)	Kahane	AJ012178
Parachlamydia acanthamoebae				
Bn$_9^T$	Amoeba/human	Amann *et al.* (1997)	Michel	AJ012179
Berg$_{1}$	Amoeba/human	Amann *et al.* (1997)	Michel	

PCR amplification and DNA sequence determination. The *rnpB* gene in species of *Chlamydiaceae* was amplified by PCR with the primer pair BH1-BH2, which was designed based on the *C. trachomatis* sequence (13) (Table 3).

Table 3. Primers used for amplification of the *rnpB* gene

Primer	Sequence	Nucleotide positions in *rnpB* of *Chlamydia trachomatis* (see Fig. 6):
BH1	5'-CGGACTTTATAAGAAAAGAT-3' (upper)	64 to 83
BH2	5'-(A/G)TAAGCCGGGTTCTGT-3' (lower)	392 to 377
BM1	5'-(A/G)(A/G)(C/A)G(A/G)(A/G)GAGGAAAGTCC-3' (upper)	48 to 64
JB1	5'-CGAACTAATCGGAAGAGTAAGGC-3' (upper)	−8 to 15
JB2	5'-GAGCGAGTAAGCCGG(A/G)TTCTGT-3' (lower)	398 to 377

The reaction mixture contained 0.2 µM of each primer, 200 µM dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 15% glycerol and 2 U *Taq* polymerase. Amplification conditions consisted of 7 cycles with 45 s at 94°C, 45 s at 42°C, 1 min at 72°C followed by 35 cycles where the annealing temperature was increased to 58 °C. If not otherwise stated, all PCR-products mentioned in the text refer to the use of the primer pair BH1-BH2, which amplified 82% of the full-length gene. To generate the 5'-flanking region from type strains of the nine *Chlamydiaceae* species we used the primer pair JB1-JB2 which was obtained from the complete RNase P RNA gene sequence of *C. trachomatis* (kindly provided by Dr J. Brown). Amplification conditions were as described for the BH1-BH2 primer pair, except that glycerol was omitted and the annealing temperatures were 53°C and 58°C, respectively. For amplification of *S. negevensis* and *P. acanthamoebae* the BH1 primer was replaced by BM1, which included highly conserved nucleotides as previously described (13), but did not permit sequencing of the 5' end of *rnpB*. The resulting PCR products were sequenced by using a terminator labelled cycle sequencing chemistry and sequence reactions were analyzed on a 310 Genetic Analyzer (Perkin-Elmer). Sequences were submitted to EMBL and all accession numbers listed in Table 2 are from the present study.

Sequence alignment and phylogenetic analysis. Sequence alignment required secondary structure modelling of each RNase P RNA molecule which was performed manually by using comparative sequence analysis. The predicted structures were subsequently used in the alignment procedure as an aid for the identification of loop and stem regions. The alignment was used to study molecular phylogeny. The calculated distance matrix was corrected for multiple base changes at single

locations by the one parameter model of Jukes & Cantor (1969)(55). This matrix was subsequently used to compute a phylogenetic tree by using the neighbor-joining program (77), implemented under the name NEIGHBOR in the Phylogenetic Inference Package, PHYLIP version 3.51c (44). Maximum parsimony trees were inferred using the DNAPARS program. The SEQBOOT program was used to bootstrap the trees based on neighbor-joining and maximum parsimony by resampling the data sets 1000 times. The construction of the maximum likelihood tree was performed with the DNAML program using the F84 model of molecular evolution applying empirical base frequencies, global rearrangement and the jumble option.

Preparation of RNase P RNA and substrates. To test *rnpB* catalytic activity, the full-length *C. trachomatis rnpB* was PCR amplified, cloned behind a T7 promoter, and assayed for tRNA precursor cleavage. We designed a PCR primer matching the 5' end (5' TTTGAATTCGAAATTAATACGACTCACTATAGCGAACTAATCGGAAGAGTA). Underlined residues match the *C. trachomatis rnpB* while the remaining part of the primer corresponds to the T7 promoter. We designed a primer complementing the 3' end (5'TTTAAGCTTGGATGGTACCTTGGAAAAGCTCGGAAGAGCGAGTAA). Underlined residues are complementary to the *C. trachomatis rnpB* and the unmarked residues were incorporated in order to be able to cleave the resulting plasmid with *Fok*I). The PCR amplified *C. trachomatis rnpB* was cut with *Eco*RI and *Hind*III and inserted into pUC19 which had been cut with the same enzymes. The recombinant plasmid was transformed in *Escherichia coli* strain DH5a following standard protocols.

The *E. coli* RNase P RNA, the *C. trachomatis* RNase P RNA and the precursor tRNA^{Tyr}Su3 were generated using the T7 DNA dependent RNA polymerase as described elsewhere (59 and references therein).

RNase P RNA assay. The RNase P RNA activity was monitored at 37 °C in our standard reaction buffer (50 mM Tris-HCl (pH 7.5), 5% (w/v) PEG 6000, 100 mM NH_4Cl (or 1M NH_4Cl as indicated) and 100 mM $MgCl_2$) as previously described (59 and references therein) and the final concentration of *C. trachomatis* RNase P RNA was ≈2.4 pmol·mL^{-1} and of precursor tRNA^{Tyr}Su3 ≈0.052 pmol·mL^{-1}.

Nucleotide sequence accession numbers. Representative nucleotide sequences for examined species have been submitted to EMBL. Accession numbers are listed in Table 2.

RESULTS

The results from Example 2 will be discussed below in association with the accompanying drawings Figs 6-8:

Fig. 6: DNA sequence comparison of *rnpB* from the 9 *Chlamydiaceae* species, *P. acanthamoebae* and *S. negevensis*. Dots indicate identity with the *C. trachomatis* A/Har-13^T sequence and dashes indicate gaps in the alignment. The hypervaribale regions P3, P12, P17 and P19 are indicated. Numbering is according to Brown (39).

Fig. 7: Deduced secondary structures of RNase P RNA in *C. psittaci, P. acanthamoebae* and *S. negevensis*. The nucleotides in the primer sequences are in lower case type, m indicates an adenine-cytosine mixture, and r indicates adenine-guanine mixture at this position. N denotes tentative nucleotides in the flanking regions of the primer based on the minimum consensus bacterial RNase P RNA (39).

Fig. 8: Neighbour-joining tree based on *rnpB*, showing the relationships among members of the family *Chlamydiaceae. P. acanthamoebae*, strain Bn9^T and *S. negevensis*, strain Z^T were chosen as outgroups. Parsimony and ML analyses produced an identical branch order. However, two taxa branched slightly different in the ML tree and this node is marked with an asterisk. Bootstrap support values at the nodes were obtained from 1000 resamplings of the data set using neighbor-joining and maximum parsimony. The scale bar indicates 5 substitutions per 100 nucleotides.

Comparison of *rnpB* sequences

PCR products that included 82% of the full-length *rnpB* gene were obtained from 60 chlamydial strains. The products from *P. acanthamoebae* strain Bn9^T and Berg17 were 313 bases long and their sequences were identical. The sequence of the 299-bp product from the Z^T strain of *S. negevensis* was 68.9% similar to the *P. acanthamoebae* sequence. The *Chlamydiaceae* PCR products were between 63.8% and 69.3% similar to the segments available from *P. acanthamoebae* and *S. negevensis*. The *rnpB* sequences from *Chlamydia* and *Chlamydophila*, which are the 2 genera in the *Chlamydiaceae*, were 75.9% - 83.3% similar. The 18 strains belonging to *Chlamydia* were >89.9% similar; the 38 strains belonging to *Chlamydophila* were >84.8% similar. The 14 *C. trachomatis* sequences differed by only a single base substitution in the LGV biovar compared to the Trachoma biovar. The 2 *Chlamydia suis* strains differed in only two nucleotide positions. The 6 *Chlamydia pecorum* strains were identical or differed by only 1 or 2 bases. Sequences were identical within species for 10 *Chlamydia psittaci* strains (except strain M56, see below), 10 *Chlamydia pneumoniae* TWAR biovar sequences, 9 *Chlamydia abortus* sequences, 3 *Chlamydia felis* sequences and the 2 *Chlamydia muridarum* sequences.

Nearly full-length gene segments (98% of the *rnpB* gene) were generated by PCR using primers JB1 and JB2 from a subset of 14 strains that included all of the 9 *Chlamydiaceae* species. Comparison of these sequences reduced the inter-species similarity as much as 2.6% as because they included the variable P3 region. The diversity in *rnpB* was large enough to clearly distinguish species groupings in the *Chlamydiaceae*. Unlike *ompA* gene products, which differ by up to 50%, and ribosomal RNA genes, which differ by <10%, this diversity will readily permit the design of genus- and group-specific PCR probes.

The strains MoPn^T (mouse) and SFPD (hamster) of the species *C. muridarum* have been shown to differ in their MOMP gene sequences (85). In contrast, the *rnpB* genes in the two *C. muridarum* strains were identical, as also has been found in the ribosomal 16S/23S intergenic spacers and 23S domain I segments (42). Evolutionary pressure on the surface exposed protein has clearly been greater than on the genes involved in the translation process.

Twenty two strains which were classified as *C. psittaci* until recently have now been separated into *C. psittaci*, *C. abortus*, *C. felis* and *C. caviae*. Our study separated these strains into the 4 species groupings by *rnpB* gene sequence differences of up to 6.7% (data not shown). These species have been isolated from host groups of distant origin and they cause a wide spectrum of diseases (Table 2). Only *C. psittaci* strain M56 conflicted with its classification as *C. psittaci* and PCR of this strain produced an *rnpB* sequence that matched the feline sequences analyzed in this study. M56 history provides some insight why this may have occurred. M56 was isolated in 1961 from a muskrat in Canada (79), then stocked and distributed to ATCC from the USDA National Animal Disease Center in Ames, Iowa, USA. In cell culture, the ATCC preparation of M56 grew out as M56 serotype in one cell line and as the feline serotype in another (Andersen, unpublished). Fukushi & Hirai (1989)(46) reported a feline serotype for M56 obtained from ATCC. PCR of M56 cultured at NADC on 8/1/90 in the yolk sacs of embryonating eggs gave *C. psittaci*-avian-like ribosomal and full-length major outer membrane protein gene sequences (42). M56 DNA used in the current study was an aliquot from the 1997 study (Table 2). In view of this history, our *rnpB* analysis suggests that M56 cultures were contaminated with feline chlamydiae during the 1960's and that uncontaminated isolates may no longer be available.

Secondary structures of the RNase P RNA

Alignment of the sequences of the *rnpB* gene derived from the nine *Chlamydiaceae* species indicated four hypervariable regions located in distinct stem loops, denoted as P3, P12, P17 and P19 in the suggested secondary structures (Fig. 7).

The P15 loop (see Fig. 7) is of interest since in most bacterial RNase P RNA molecules it harbours a GGU-motif that interacts with tRNA precursors by base pairing with the 3'-terminal RCCA motif of the tRNA (60). The absence of this sequence motif from all members of the *Chlamydiales* is striking and an ATAA-bulge is seen in all nine *Chlamydiaceae* species (positions 291 to 294 in *C. psittaci,* Fig. 7), except in *C. pneumoniae* (GAAA) and in *C. felis* (ACAA). Furthermore, a different structure in the P15 region of *Chlamydiaceae* species is rationalized by the finding that none of the identified tRNA genes encode the 3' terminal CCA sequence (80).

Interestingly, the P15 region in *P. acanthamoebae* harbours a purine rich bulge carrying a GGU-motif. This might indicate that these RNase P RNAs interact with the 3' terminal RCCA sequence as does *E. coli* RNase P RNA, given that the CCA sequence is encoded in the tRNA genes in this species. By contrast, the P15 loop structure of RNase P RNA derived from *S. negevensis* is similar to that observed in most cyanobacteria (87) and it carries a GGAU-motif in the P15 loop as does RNase P RNA derived from *Thermus thermophilus* (53). It has been suggested that this loop of RNase P RNA in *T. thermophilus* carries a high-affinity binding site for the 3' end of a tRNA precursor (52) and it may therefore also be valid for *S. negevensis.*

The RNase P RNA in *Chlamydiaceae* species harbour a P18 helix while *P. acanthamoebae* and *S. negevensis* appears to lack this element. It has previously been shown that the P18 helix can be deleted without losing catalytic activity, suggesting that it is not directly involved in catalysis (49). The P18 helix, when present, is associated with a phylogenetically conserved GNRA tetra loop which docks into its suggested receptor in P8 (the G83C93 base pair in *C. psittaci*; Fig 2; (38, 62). Furthermore, bacterial RNase P RNAs that lack the P18 helix have an extended P8 helix and it has been suggested that this compensates for the loss of P18 (38). Since neither *P. acanthamoebae* nor *S. negevensis* have an extended P8 or an apparent P18 with a GNRA tetra loop, perhaps the nucleotides in the P18 region form an alternative structural element that interacts with P8.

A long range interaction has also been suggested between the GNRA tetra loop in the P14 helix and the P8 stem (38, 62). This is supported by our present data in all nine species of *Chlamydiaceae* (the U82A94 base pair and G201 in *C. psittaci,* Fig. 7) and by the presence of an A in the P14 loop and the GC base pair in P8 in *P. acanthamoebae* and *S. negevensis* (Fig. 2). Given the presence of this interaction, it is surprising that in the three serovars L1 to L3 of *C. trachomatis* the G205 nucleotide (corresponding to G201 in *C. psittaci* in Fig. 7) is substituted by an A, but without a corresponding base pair shift in the P8 helix.

In the minimum consensus bacterial RNase P RNA certain positions have 100% conserved nucleotide bases (39). Our data showed that the well-conserved cytosine at position 60 has been replaced by a uracil in all examined species of the *Chlamydophila* genus (Fig 7), while no change was observed for the *Chlamydia* genus. This generates either a UG wobble base pair or a UA base pair, depending on the residue at postion 376 (numbering refers to *C. psittaci*). The region of the RNase P RNA derived from these species could not be examined with the primers used in the present study.

Cleavage of tRNA precursors by *C. trachomatis* RNAse P RNA
Bacterial RNase P RNA is catalytically active in the absence of the RNase P protein moiety (33 and references therein). To investigate whether *Chlamydia* RNase P RNA alone is able to cleave its substrate we generated *C. trachomatis* RNase P RNA and analysed the cleavage pattern using the *E. coli* $tRNA^{Tyr}Su3$ (pSu3) precursor as substrate. This RNase P RNA was indeed able to cleave pSu3 at the expected position only when using NH_4Cl at high concentration, as described in Methods. This is in keeping with a previous observation of cleavage by *C. trachomatis* RNase P RNA (51). Taken together with the structural observations this demonstrates that RNase P RNA does not require a P15 internal loop (or a P15 hairpin loop) for catalytic activity. However, we note that the extent of cleavage by *C. trachomatis* RNase P RNA was significantly reduced compared to cleavage by *E. coli* RNase P RNA.

Phylogeny of the family *Chlamydiaceae*
The secondary structures of the helices P15, P16, P17, P18, and P19 were the most difficult regions to resolve for the members of the *Chlamydiaceae*. Consequently, two of these regions, namely P17 and P19, were removed from the final data set that was used for phylogenetic calculations. This was due to the high nucleotide variability in the locale of P17 and the apparent absence of the helix P19 in the *rnpB* gene of *P. acanthamoebae* (Figs 6 & 7). Also, the ambiguously aligned positions 94, 150, 151, 153, 286 and 298 (according to the numbering of the *rnpB* gene of *C. trachomatis* in Fig. 6) were removed prior to phylogenetic analysis. Gapped positions were generally not omitted from the final alignment except for the termini of the 5'-end, since the positions 1 to 68 were not determined for *S. negevensis* and *P. acanthamoebae*. Consequently, the corrected final alignment comprised 271 positions.

Different algorithms were used to calculate evolutionary trees to reveal the phylogenetic relationships among the species of the family *Chlamydiaceae*. Virtually identical tree topologies were obtained by using distance matrix and character based methods. A representative phylogenetic tree derived by using neighbor-joining (NJ) (Saitou & Nei, 1987) is shown in Fig. 3. The stability of the branching order was

evaluated statistically by the determination of bootstrap percentage values. These values as obtained by NJ and maximum parsimony are given at the nodes. The branching order supported by the maximum likelihood tree (ML) has also been added to each branching point in Fig. 3. Two taxa branched somewhat differently in the dendrogram constructed by ML and the actual node displaying this instability has been furnished with an asterisk. Identical tree topologies to those shown in Fig. 3 were also obtained when only using *rnpB* data from the species belonging to *Chlamydophila* and *Chlamydia* but extending the data set to comprise the nucleotide information of the 5'-end. Therefore, the branching order in this part of the tree was not resolved.

The tree in Fig. 8 shows that the genera *Chlamydophila* and *Chlamydia* can readily be distinguished from one another by comparing *rnpB* gene sequences. These findings are consistent with recently published phylogenies based on full-length 16S and 23S ribosomal RNA genes, and on their intergenic spacer regions (42, 43, 73). However, this conflicts with the results presented for the 16S rRNA gene by Pettersson *et al.* (1997). A plausible explanation is that the 16S rRNA study was based on only 4/5 of the full-length nucleotide information for these genes and that some of the sequences used for comparison were rather distantly related. Therefore, some phylogenetic information was lost in the final data set. In a subsequent 16S rRNA analysis using almost complete 16S rRNA gene sequences and only close relatives as outgroups, there was limited correlation with other branching orders. Thus it can be concluded that 16S rRNA genes provide low resolution in describing the evolutionary interrelationships of the members of the *Chlamydiaceae* family.

Analysis of the *rnpB* gene demonstrated that chlamydial phylogeny need not rely entirely on genes for which there is only weak branch support. While 16S rRNA analyses show numerous long branches with clusters attached, the *rnpB* analysis has evenly distributed sequence differences that distinguish chlamydial groups at family, genus, and species levels. The sequence diversity of the *rnpB* gene in the species of the order *Chlamydiales* will make it possible to use this gene for the discrimination of chlamydial species. Moreover, this specificity reveals a functional isolation of each grouping that is consistent with the species-specific ecological niches as described (70). The conservation is also consistent with previously identified groupings. The specificity found in so basic a function as tRNA processing, suggests that the species groupings in the *Chlamydiaceae* have been evolutionarily isolated for a very long period of time. The presented phylogenetic analysis supports the revision of the classification of the *Chlamydiaceae* family as previously described (43).

In summary, the sequence of the RNase P RNA gene (*rnpB*) was determined for 60 strains representing all nine species in the

Chlamydiaceae family and for the related *Chlamydiales* species, *Parachlamydia acanthamoebae* and *Simkania negevensis*. These sequences were used to infer evolutionary relationships among the *Chlamydiaceae*. The analysis separated *Chlamydophila* and *Chlamydia* into two lineages, with *Chlamydophila* forming three distinct clusters: the *Chlamydophila pneumoniae* strains, the *Chlamydophila pecorum* strains and a third cluster comprising the species *Chlamydophila psittaci, Chlamydophila abortus, Chlamydophila caviae* and *Chlamydophila felis*. The *Chlamydia* line of descent contained two clusters, with the *Chlamydia suis* strains distinctly separated from strains of *Chlamydia trachomatis* and *Chlamydia muridarum*. This analysis indicated that the *rnpB* sequence and structure are distinctive markers for species in the *Chlamydiaceae*. We also demonstrated that the RNase P RNA derived from *C. trachomatis* is able to cleave a tRNA precursor in the absence of protein. Our findings are discussed in relation to the structure of *Chlamydia* RNase P RNA.

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CLAIMS

1. A method for detection of pathogenic organisms including inter-species differentiation, comprising analysis of the P3 and/or P19 hypervariable region(s) of the RNase P RNA gene.

2. A method according to claim 1, comprising nucleic acid amplification.

3. A method according to claims 1-2, wherein the nucleic acid is sequenced for species identification.

4. A method according to claims 1-2, wherein the nucleic acid is finger printed for species identification, such as by RFLP, heteroduplex analysis, size determination, melting point determination etc.

5. A method for detection of pathogenic organisms including inter-species differentiation, comprising amplification of nucleic acids of hypervariable region(s) of the RNase P RNA gene from the pathogens; forming a heteroduplex with related nucleic acid; and analysis thereof.

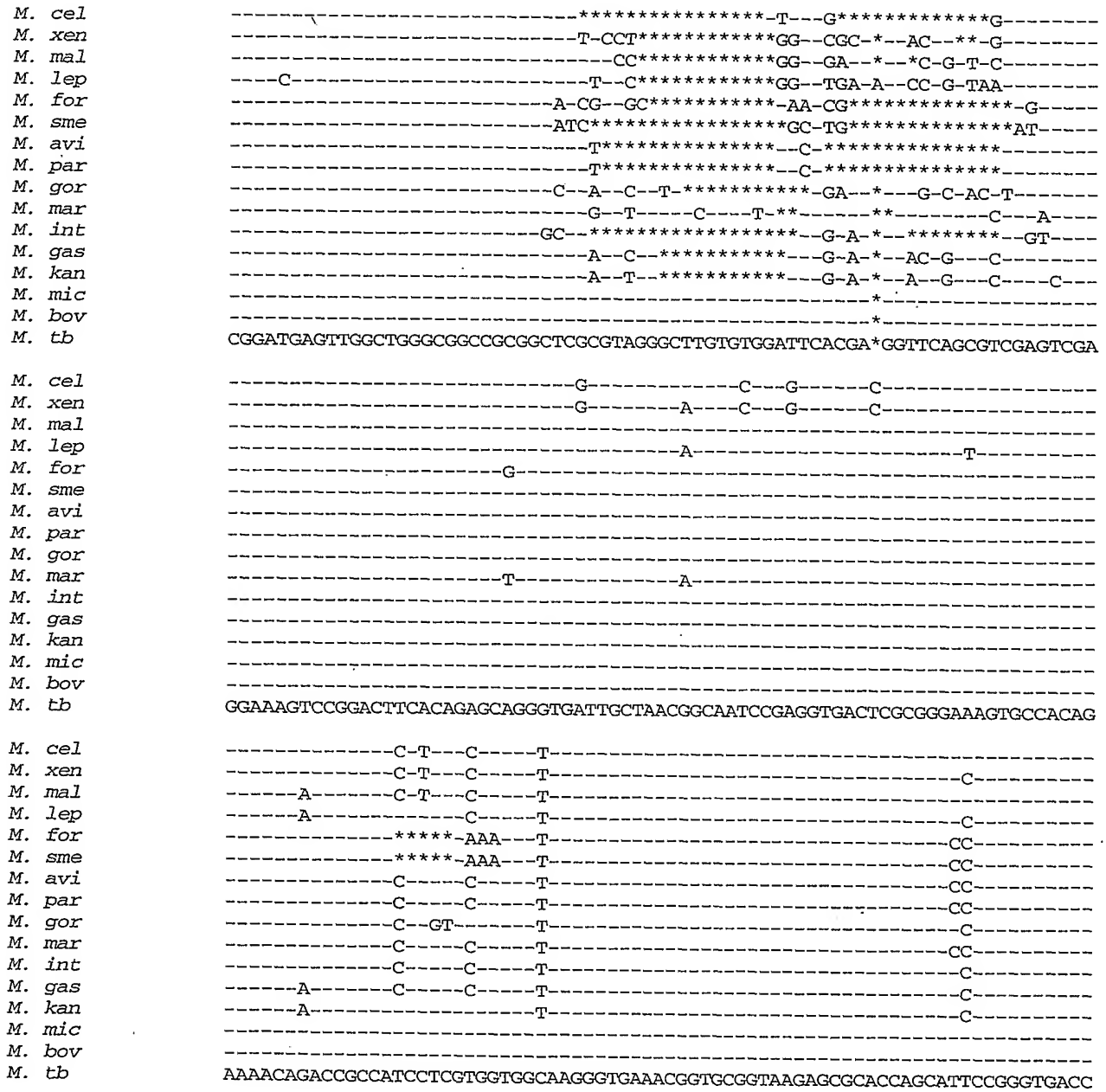
6. A method according to claim 6, wherein the hypervariable region is P3 and/or P19.

7. A method according to any of the above claims, wherein the pathogens are mycobacteria or chlamydia.

8. A method according to claim 7, wherein the pathogens are chlamydia and primers used for amplification are JB1 and JB2 (Table 3).

9. Use of the P3 and/or P19 variable regions(s) in the RNAse P RNA gene as a drug target in the production of a medicament for the treatment of microbial infection.

Fig. 1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M. kan
M. mic
M. bov
M. tb
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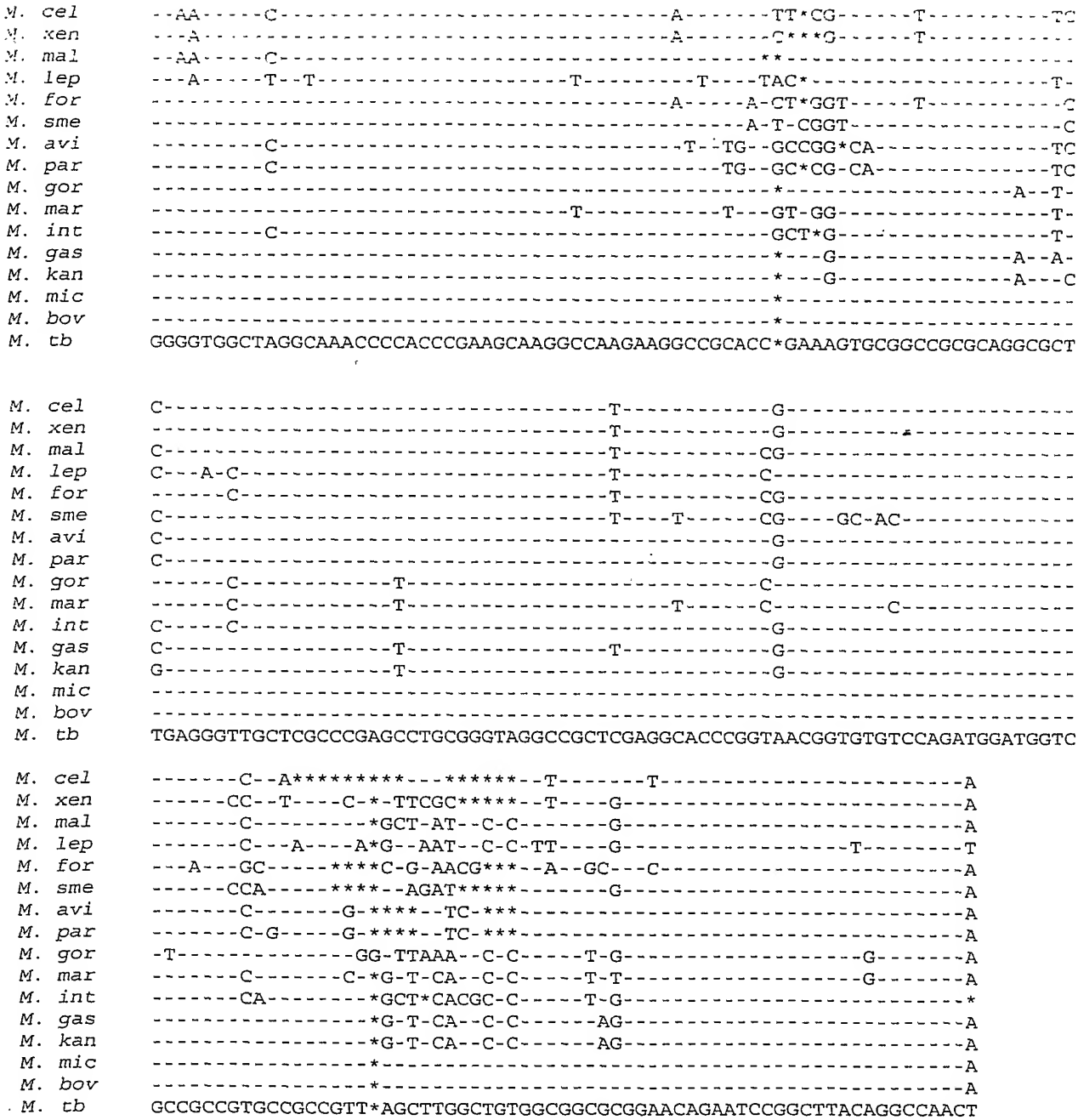
Fig. 1 cont.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M. xen ---A----------A-------C***G------T----------
M. mal --AA-----C----------**----------
M. lep ---A-----T--T----------T---------T----TAC*----------T-
M. for ----------A-----A-CT*GGT-----T----------C
M. sme ----------A-T-CGGT----------C
M. avi ---------C----------T--TG--GCCGG*CA----------TC
M. par ---------C----------TG--GC*CG-CA----------TC
M. gor ----------*----------A--T-
M. mar ----------T----------T---GT-GG----------T-
M. int ---------C----------GCT*G----------T-
M. gas ----------*---G----------A--A-
M. kan ----------*---G----------A---C
M. mic ----------*----------
M. bov ----------*----------
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M. cel C----------T----------G----------
M. xen ----------T----------G----------
M. mal C----------T----------CG----------
M. lep C---A-C----------T----------C----------
M. for ------C----------T----------CG----------
M. sme C----------T----T------CG----GC-AC----------
M. avi C----------G----------
M. par C----------G----------
M. gor ------C----------T----------C----------
M. mar ------C----------T----------T------C---------C----------
M. int C-----C----------G----------
M. gas C----------T----------T----------G----------
M. kan G----------T----------G----------
M. mic ----------
M. bov ----------
M. tb TGAGGGTTGCTCGCCCGAGCCTGCGGGTAGGCCGCTCGAGGCACCCGGTAACGGTGTGTCCAGATGGATGGTC
M. cel -------C--A*********---*******--T-------T----------A
M. xen ------CC--T----C-*-TTCGC******--T----G----------A
M. mal -------C---------*GCT-AT--C-C-------G----------A
M. lep -------C---A----A*G--AAT--C-C-TT----G----------T---------T
M. for ---A---GC-----****C-G-AACG****--A--GC---C----------A
M. sme ------CCA-----****--AGAT*****-------G----------A
M. avi -------C-------G-*****--TC-***----------A
M. par -------C-G-----G-*****--TC-***----------A
M. gor -T--------------GG-TTAAA--C-C-----T-G----------G-------A
M. mar -------C-------C-*G-T-CA--C-C-----T-T----------G-------A
M. int -------CA--------*GCT*CACGC-C-----T-G----------*
M. gas ----------*G-T-CA--C-C------AG----------A
M. kan ----------*G-T-CA--C-C------AG----------A
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Fig. 2

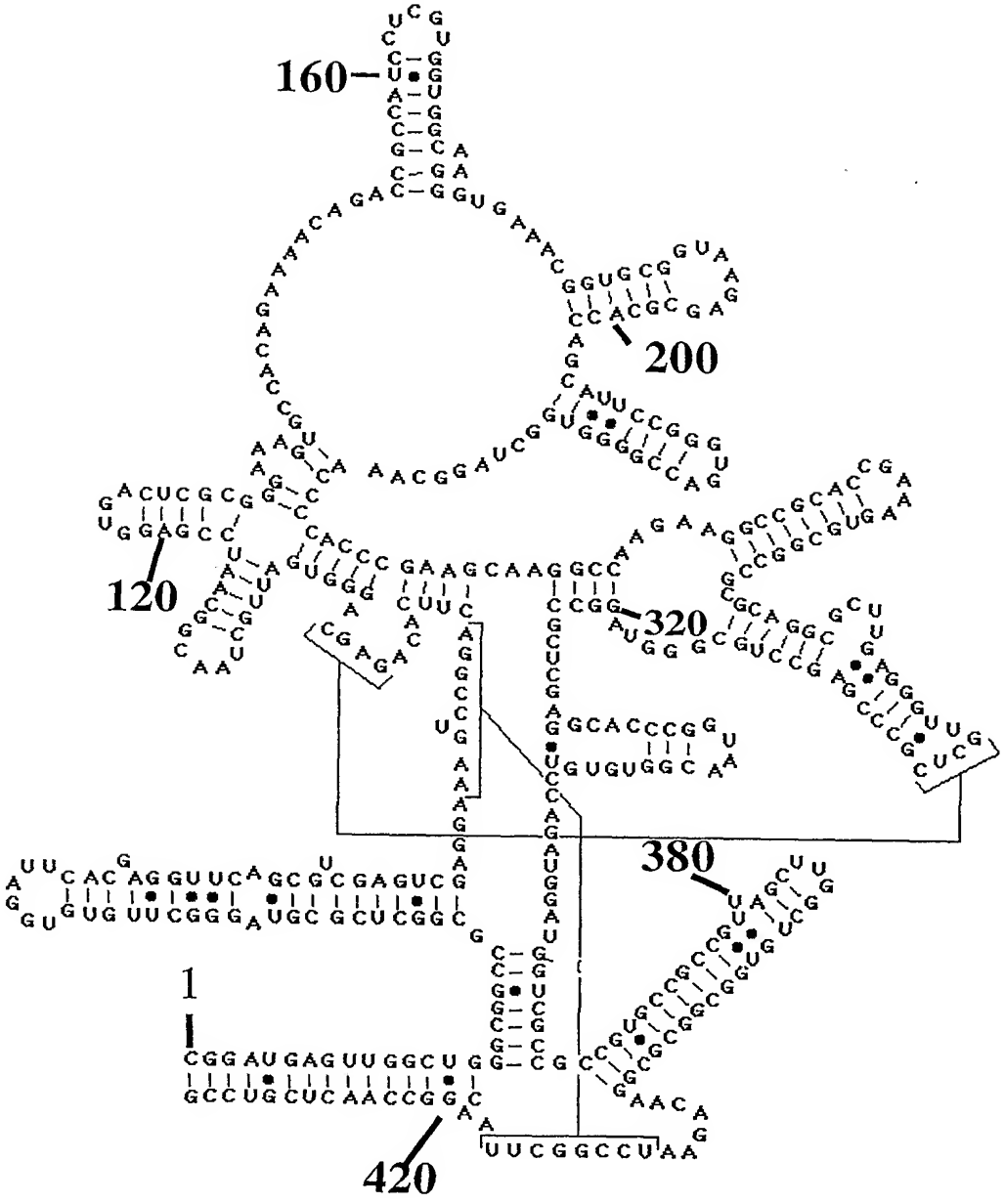

Fig. 3

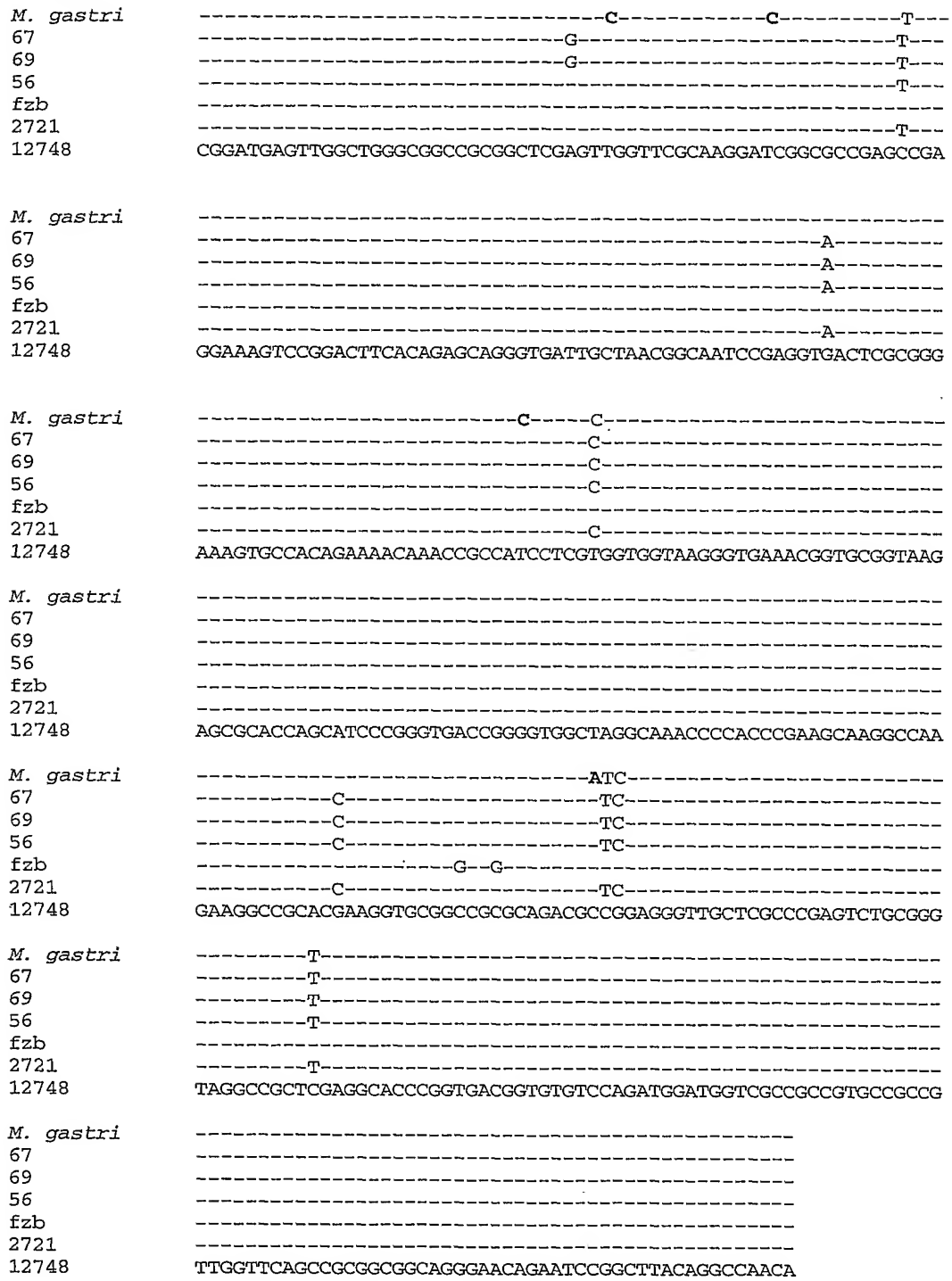
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Fig. 4A

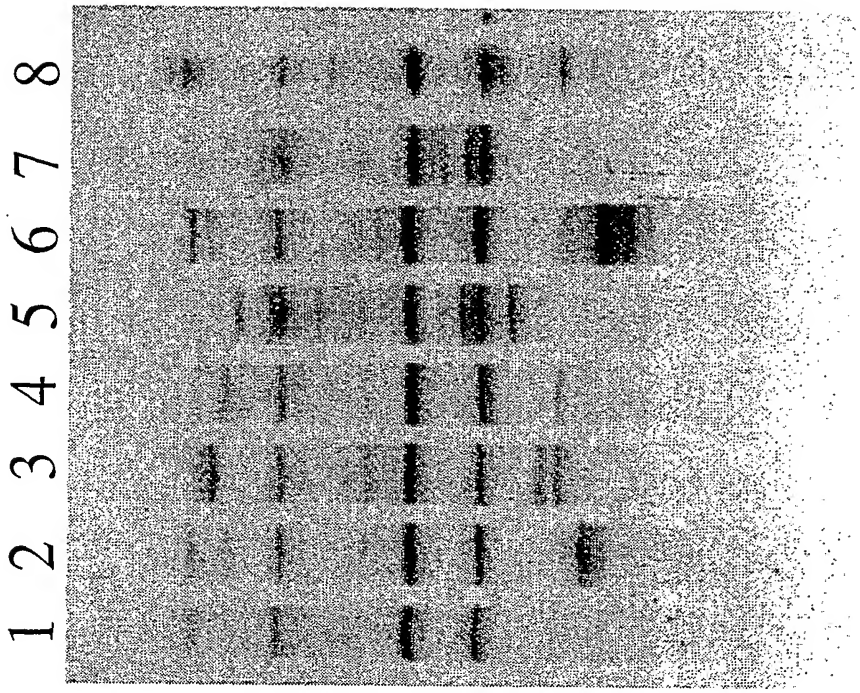


Fig. 4B

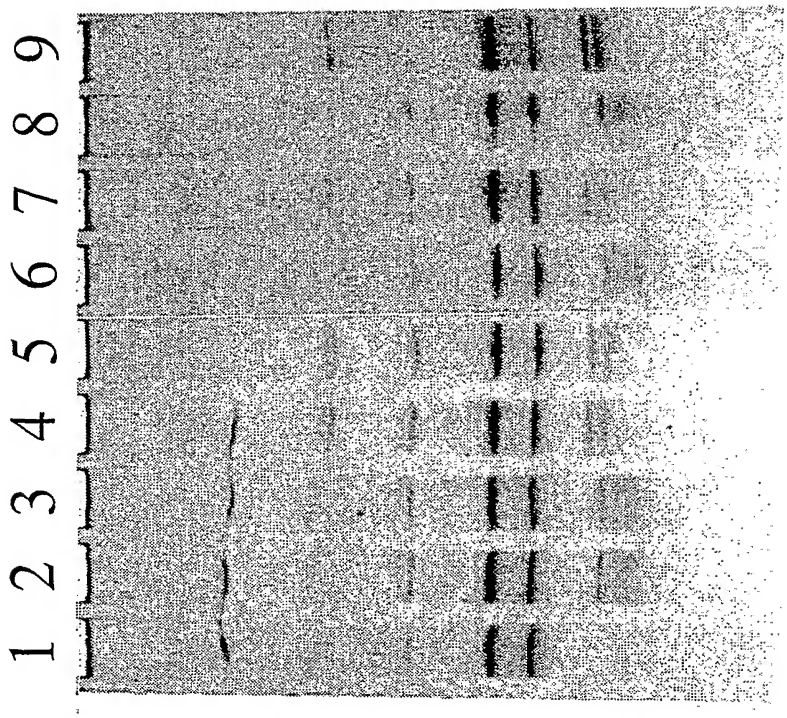
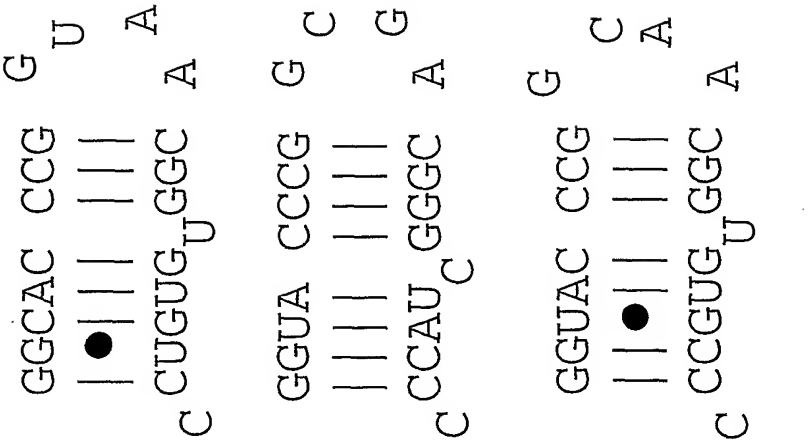


Fig. 5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M. marinum

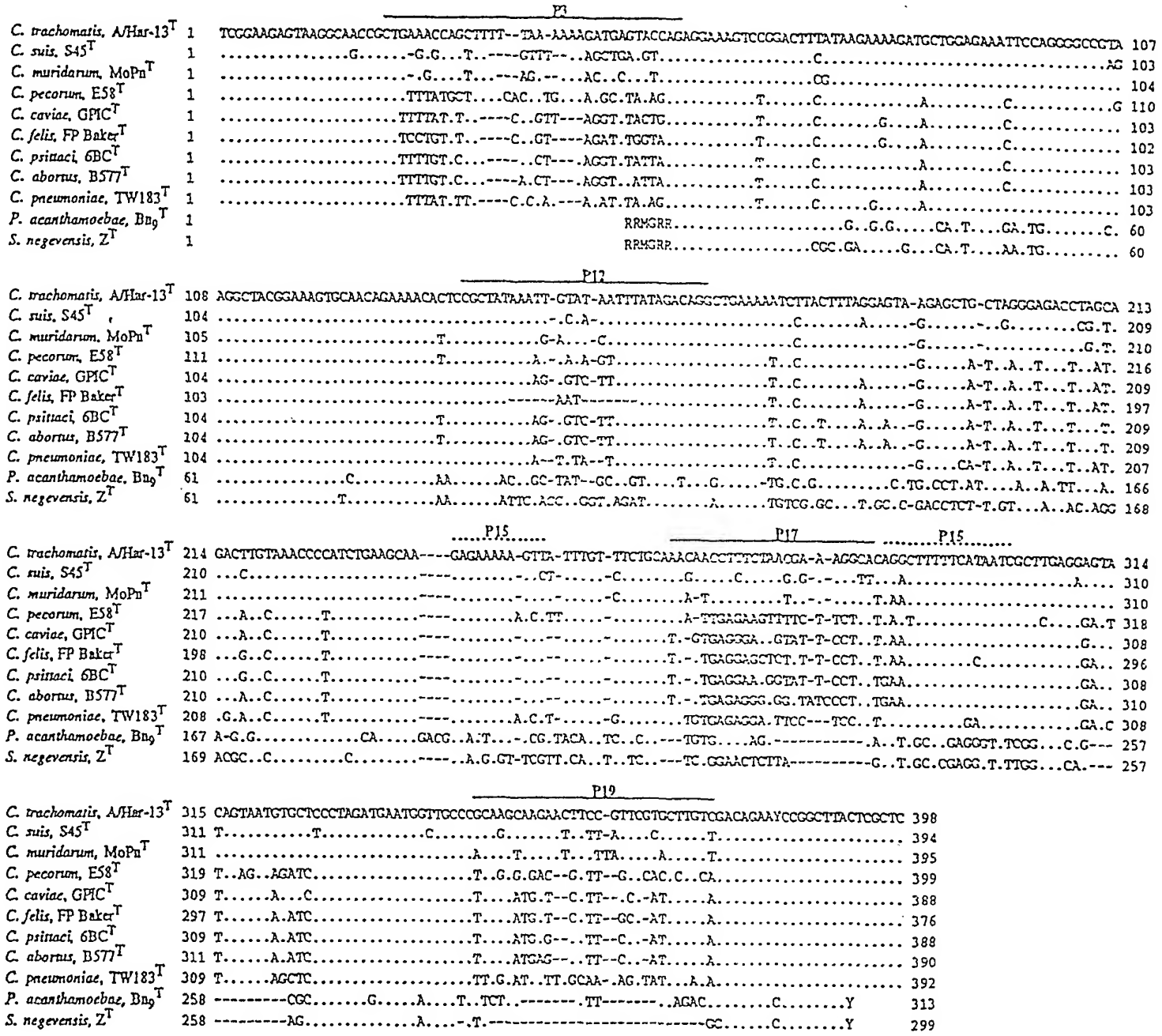
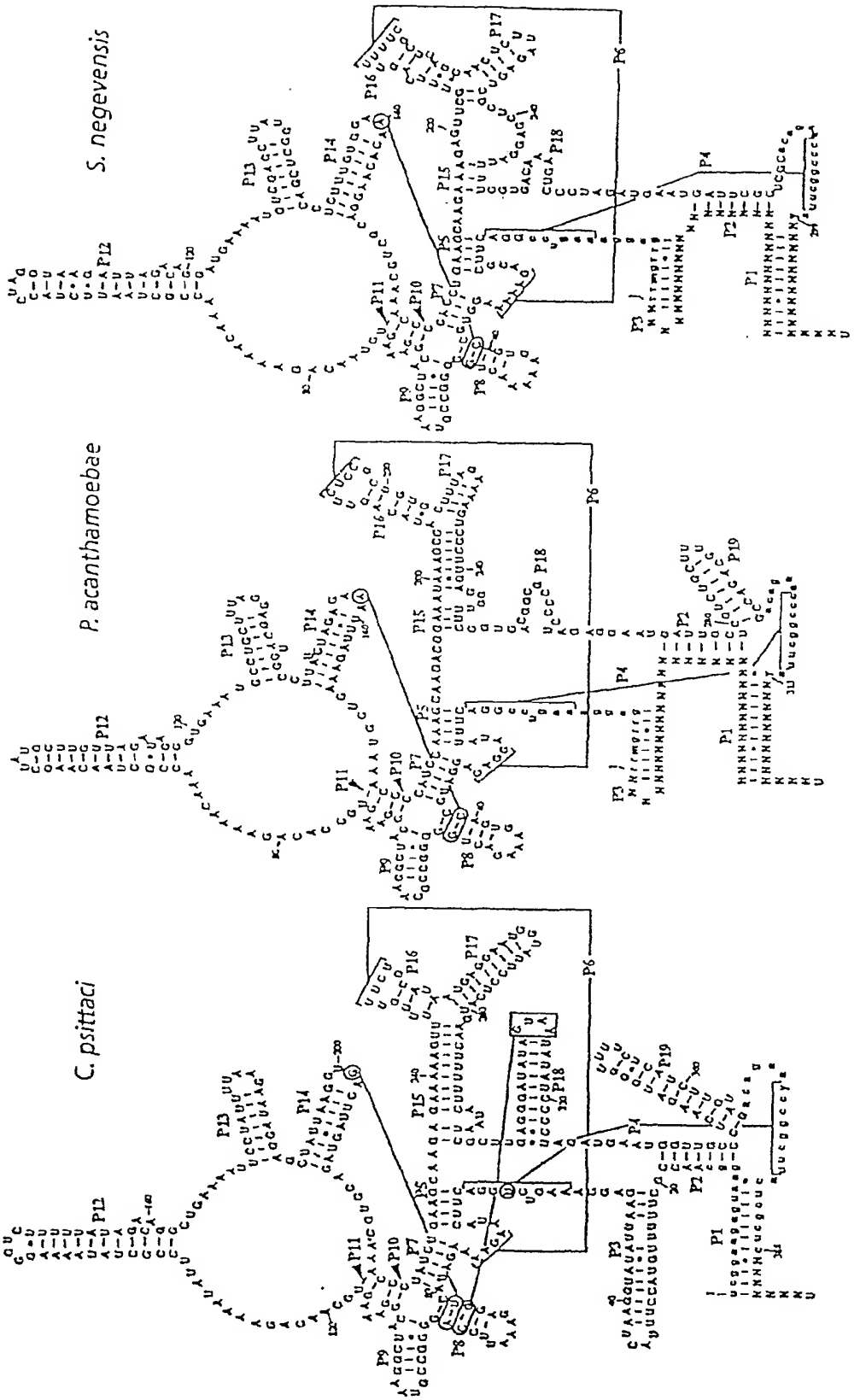


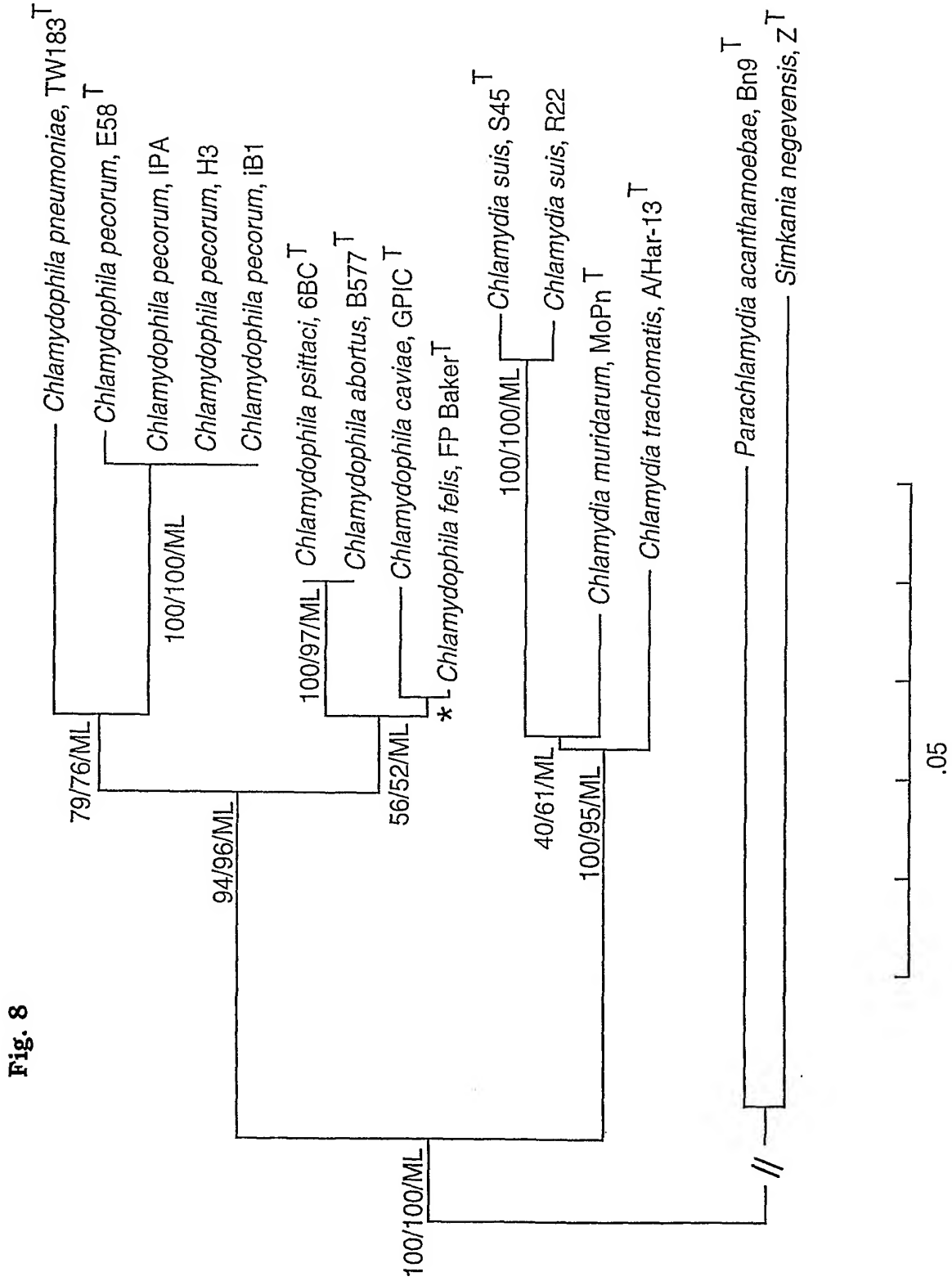
Fig. 6

SUBSTITUTE SHEET (RULE 26)

8/10

Fig. 7

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Chlamydia suis, R22
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Parachlamydia acanthamoebae, Bn9 T
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Fig. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 01/00031

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/68, G01N 33/569
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5574145 A (T.G. BARRY ET AL), 12 November 1996 (12.11.96), column 2, line 57 - column 3, line 13; column 8, line 42 - line 52, abstract ---	1-8
A	WO 9911653 A1 (SMITHKLINE BEECHAM CORPORATION), 11 March 1999 (11.03.99), page 40, line 1 - line 24 ---	1-8
A	HAAS, E.S. et al. "SURVEYE AND SUMMARY, Evolutionary variation in bacterial RNase P RNAs". Nucleic Acids Research, 1998, Vol. 26, No. 18, pages 4093 - 4099. See page 4095, column 2, line 5 - line 7; page 4096, column 1, line 40 - line 55 ---	1-8

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 2001	17 -04- 2001
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Frida Plym Forshell /OGU Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 01/00031

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JAMES, B.D. et al. "The Secondary Structure of Ribonuclease P RNA, the Catalytic Element of a Ribonucleoprotein Enzyme". Cell, January 1988, Vol. 52, pages 19 - 26. See page 22, column 2, line 1 - line 9, figure 3	1-8

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE01/00031

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 9
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see extra sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Claim 9, relating to use of variable regions in the RNase P RNA gene as drug targets, fails to comply with the prescribed requirements in PCT Article 6. The claim is not clear and does not define the matter for which protection is sought. A meaningful search could therefore not be carried out.

INTERNATIONAL SEARCH REPORT

Information on patent family members

25/02/01

International application No.
PCT/SE 01/00031

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5574145 A	12/11/96	AT 150091 T	15/03/97
		AU 630932 B	12/11/92
		AU 5365290 A	25/10/90
		DE 69030131 D,T	17/07/97
		DK 395292 T	15/09/97
		EP 0395292 A,B	31/10/90
		SE 0395292 T3	
		ES 2100161 T	16/06/97
		GR 3023600 T	29/08/97
		IE 81145 B	03/05/00
		JP 3097059 B	10/10/00
		JP 3130099 A	03/06/91
		ZA 9002924 A	27/02/91
WO 9911653 A1	11/03/99	EP 1019431 A	19/07/00